Biosynthesis of 6a-Hydroxypterocarpans: Deuterium NMR Evidence for Direct Hydroxylation of Pterocarpans

Stephen W. Banks and Paul M. Dewick

Department of Pharmacy, University of Nottingham, Nottingham, NG7 2RD, UK

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^{2}H-NMR analysis of the 6a-hydroxypterocarpan phytoalexin pisatin derived in $CuCl_2$-treated pea (*Pisum sativum*) pods from [6,11a-$^2H_2$]maackiain has demonstrated the retention of all ^{2}H labels. This establishes that no pterocarp-6a-ene or pterocarp-6-ene intermediate is involved, and thus confirms a direct 6a-hydroxylation mechanism for the biosynthesis of 6a-hydroxypterocarpans from pterocarpans.

Introduction

(+)-Pisatin (**3**) is a 6a-hydroxypterocarpan phytoalexin produced by pea (*Pisum sativum*) tissues on fungal infection or abiotic treatments [1]. From the results of feeding experiments using radiochemically-labelled precursors [2, 3], the biosynthetic pathway to pisatin has been shown to proceed via the pterocarpan (+)-maackiain (**1**), conversion into (+)-6a-hydroxymaackiain (**2**) and then methylation to yield pisatin (**3**). The transformation of maackiain to 6a-hydroxymaackiain could be achieved by the following mechanisms (Fig. 1):

(a) direct 6a-hydroxylation probably involving a mono-oxygenase enzyme,
(b) dehydrogenation/hydration involving a pterocarp-6a-ene intermediate (**4**), or
(c) dehydrogenation/hydration involving a pterocarp-6-ene intermediate (**5**).

The poor incorporations of the pterocarp-6a-enes anhydropisatin and dehydromaackiain (**4**) into pisatin [2] suggest however that mechanism (b) is unlikely to operate, and overall, results [3] tend to favour mechanism (a), *i.e.* direct hydroxylation. To investigate further the nature of the mechanism, we

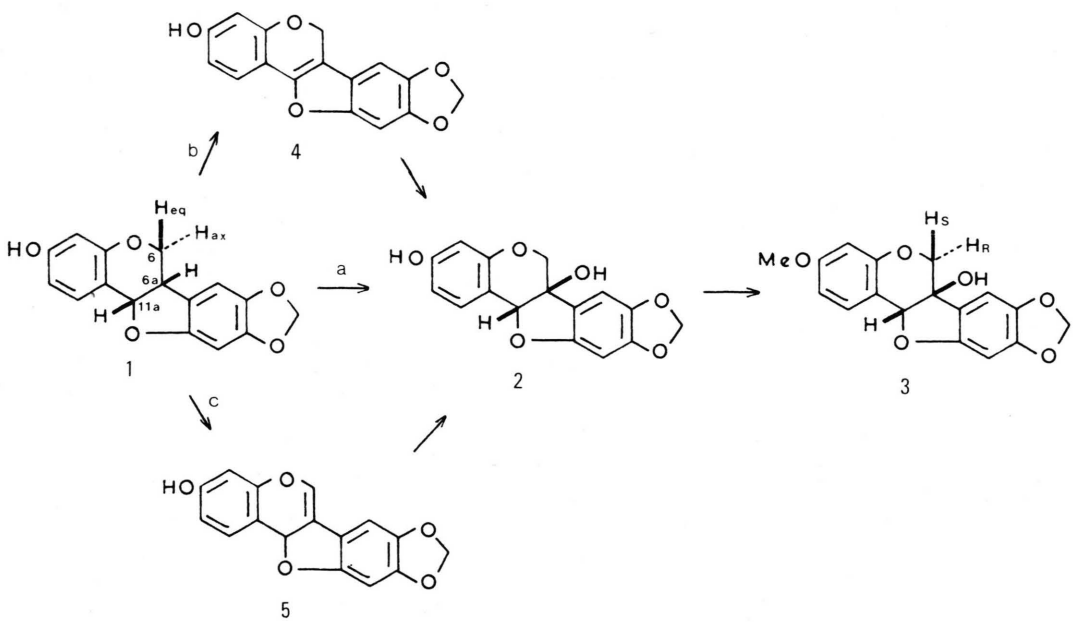

Fig. 1. Probable mechanisms for biosynthesis of (+)-pisatin (**3**) from (+)-maackiain (**1**).

Reprint requests to Dr. Dewick.

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have synthesised (±)-[6,11a-$^2H_2$]maackiain and observed its incorporation into pisatin in $CuCl_2$-treated pea pods. Analysis of the labelled pisatin by deuterium NMR establishes beyond doubt that no pterocarpene intermediate is involved, and confirms a direct hydroxylation mechanism.

Experimental

Synthesis of (±)-[*6,11a-*$^2H_2$]*maackiain*

Sodium borodeuteride (500 mg, 98% ^2H) was added in portions over 2 h to a stirred solution of 2′,7-dihydroxy-4′,5′-methylenedioxyisoflavone [4] (280 mg) in EtOH (Analar, 30 ml) and dry THF (30 ml). The mixture was stirred overnight at room temperature, concentrated under vacuum, treated with HCl (10%, 10 ml), then extracted with EtOAc (3×25 ml). The combined extracts were washed with H_2O, then evaporated. (±)-[6,11a-$^2H_2$]Maackiain was isolated by TLC (Merck TLC-Kiesel gel $60GF_{254}$, hexane-acetone, 2:1) and recrystallised from aqueous MeOH, yield 110 mg.

Comparison of its ^1H NMR spectrum (250 MHz, $(CD_3)_2CO$, TMS) with that of [^1H]maackiain [5] showed the signal for H-11a (δ 5.49, d, J = 6.7 Hz) to be reduced in intensity to approximately 4% of normal, indicating 96% deuteriation at this position. The signal for H-6eq at δ 4.28 (m, approx. dd, J = 9.6, 3.3 Hz) became a less-resolved multiplet of intensity 53% of normal, thus showing 47% deuteriation. Signals for H-6ax (δ 3.63, approx. t, J = 10.0 Hz) and H-6a (δ 3.56, m) overlap, and the relative proportions of deuteriation could thus not be assessed accurately. The overall integral was reduced to 57% of normal, thus corresponding to 43% deuteriation at H-6ax assuming no deuteriation at H-6a (confirmed by the ^2H NMR spectrum, see Results). As expected, therefore, position 11a was almost completely deuteriated (ca. 96%), and the two 6 hydrogens were equally labelled to an extent of ca. 45% (maximum possible 50%). In a second synthesis, the amount of labelling was somewhat lower, ca. 84% at position 11a, and 40% at each 6 hydrogen, indicating exchange of the borodeuteride with solvent hydrogen.

Biosynthesis of pisatin

Pea pods were obtained from plants (cv. Kelvedon Wonder) grown under normal garden conditions. Aqueous $CuCl_2$ (3×10^{-3} M) was injected into mature pods (ca. 300, 1.4 kg), which were then left for 12 h in the light under conditions of high humidity. Unabsorbed $CuCl_2$ was removed by syringe, and a solution of (±)-[6,11a-$^2H_2$]maackiain (215 mg, ^2H-content ca. 90% at H-11a, 42% at each H-6) and (±)-[11a-^{14}C]maackiain [2] (1.57 mg, specific activity 2.15×10^8 dpm/mM), as the sodium salts in phosphate buffer (0.1 M, pH 7.0, 770 ml), was injected. This volume of feeding solution was equivalent to the volume of inducing solution used. The pods were then left for 36 h in a 12 h dark − 12 h light regimen in the same humid conditions. After this time, the plant tissue was worked up by homogenising with cold EtOH (2*l*) in a blendor. The mixture was filtered, and the solids extracted with two further 1*l* portions of cold EtOH. Pisatin was isolated and purified from the combined extracts by the same procedure as reported previously [2]. The yield of pisatin after TLC purification was 75 mg, total pisatin content before purification being 84 mg. Specific activity of the product was 4.04×10^4 dpm/mM, corresponding to a dilution value of 38.6 (*i.e.* specific incorporation 2.6%), and an incorporation of 0.95%.

Results and Discussion

The deuterium NMR spectrum (38.4 MHz, $(CH_3)_2CO$) of the [6,11a-$^2H_2$]maackiain fed (Fig. 2A) confirmed the data from the proton spectrum and showed three signals at δ 5.47, 4.25 and 3.62, corresponding to H-11a, H-6eq and H-6ax respectively, and the relative intensities were 2.2:1:1, in good agreement with the proton spectrum. When this material was fed to pea pods, the labelled pisatin produced showed a deuterium spectrum (acetone solution) as in Fig. 2B. This contained two signals at δ 5.26 and δ 4.09, with relative intensities of approximately 1:1. The two signals correspond to H-11a and H-6 respectively, and ^1H NMR confirmed that the two H-6 proton resonances are indeed coincident in acetone solution. Comparison of the peak areas with that of the natural abundance solvent peak indicated a deuterium content for each signal of approximately 2.0%, in good agreement with that calculated from the specific incorporation, *i.e.* 2.6×0.90 = 2.3%. In chloroform solution, the two H-6 protons are clearly resolved [6] and the deuteriated sample then showed in its deuterium spectrum (Fig. 2C) the expected three signals at δ 5.27,

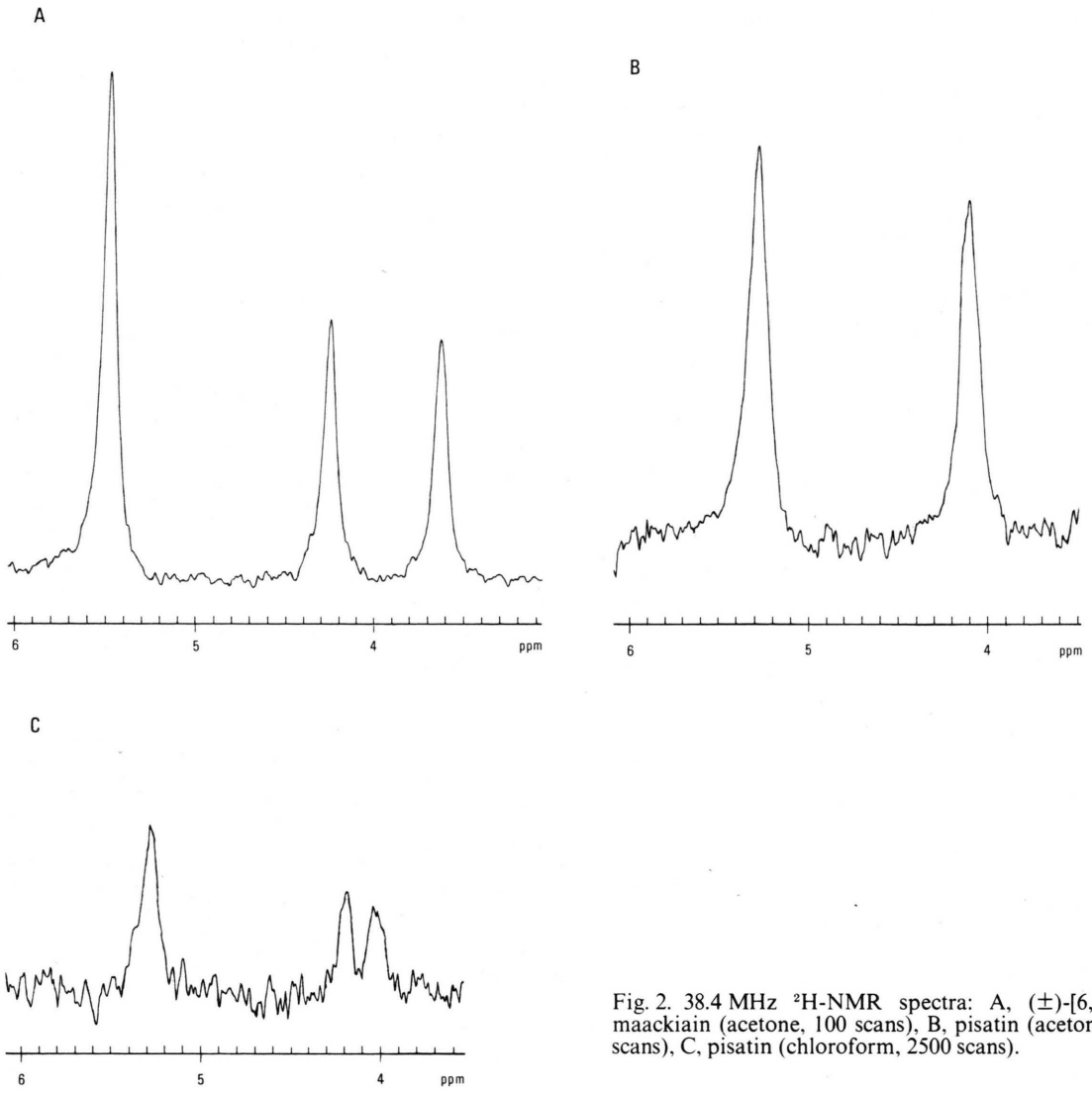

Fig. 2. 38.4 MHz ^{2}H-NMR spectra: A, (±)-[6,11a-$^{2}H_{2}$]-maackiain (acetone, 100 scans), B, pisatin (acetone, 11 000 scans), C, pisatin (chloroform, 2500 scans).

4.19 and 4.04 corresponding to H-11a, H-6s and H-6R respectively. Within the limits of experimental error, the relative intensities were essentially identical (2:1:1) to those of the precursor maackiain.

Since pisatin produced biosynthetically from the labelled maackiain contains deuterium at position 11a and both 6 positions, and furthermore retains the same relative proportions of deuterium label, the intermediacy of pterocarp-6a-enes or pterocarp-6-enes can definitely be excluded. The involvement of either would necessitate loss of label from positions 11a or 6, and thus the relative enrichment would change markedly. The slight loss of deuterium from 11a as indicated by the intensity measurements is not regarded as real, but probably reflects the lower levels of accuracy attainable in deuterium NMR studies because of the low sensitivity problems. Total deuterium content was very similar to that calculated from ^{14}C incorporations, and also indicates no overall loss of deuterium label. Thus, the most likely biosynthetic pathway to pisatin involves direct 6a-hydroxylation of maackiain, and it is very possible that a monooxygenase enzyme could be involved. This is entirely in keeping with the observation that the hydroxylation is found to proceed with retention of configuration at C-6a [3].

Similar 6a-hydroxylation of pterocarpans is observed in the sequences employed by several microorganisms for the metabolic detoxification of pterocarpan phytoalexins [7]. These hydroxylations are also thought to be catalysed by mono-oxygenase enzymes [8], and similarly, where evidence is available [9], proceed with retention of configuration.

Feeding experiments have shown that both (+)- and (−)-isomers of maackiain may be used by pea tissue in the biosynthesis of pisatin, but (−)-maackiain is transformed into (−)-pisatin rather than the normal (+)-isomer [5]. Since (±)-maackiain was fed in this experiment it is thus likely that a proportion of (−)-pisatin was also present in the final metabolite. This, however, in no way affects the conclusions discussed, since the pathway to (−)-pisatin appears to parallel that to (+)-pisatin [3].

The equivalence or non-equivalence of NMR signals for the 6 protons of 6a-hydroxypterocarpans has been discussed [9] and was regarded as purely accidental. Clearly, the chemical shifts of the 6 protons of pisatin are solvent dependent, and this is likely to hold also for other 6a-hydroxypterocarpans. It may be valuable therefore not to restrict NMR studies of suspected 6a-hydroxypterocarpans to a single solvent system.

The production of pisatin in these studies (ca. 60 μg/g fresh wt. pea pods) was markedly lower than in previous feeding experiments (ca. 160 − 200 μg/g) [2, 3, 5]. The cause of this is not entirely certain, but reduced levels have been observed in a number of similar experiments where relatively large amounts of maackiain have been fed [10]. Some pterocarpan phytoalexins, *e.g.* pisatin, phaseollin and glyceollin are known to produce phytotoxic effects [11] and it is probable that maackiain has similar properties. Thus the presence of relatively large amounts of maackiain in these experiments may have an inhibitory effect on biosynthetic processes, albeit to related phytoalexins.

Acknowledgement

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